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Use of the Short-term Inflammatory Response in the Mouse Peritoneal Cavity to Assess the Biological Activity of Leached Vitreous Fibers

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We used a special-purpose glass microfiber sample, Johns-Manville Code 100/475, to study the effects of various acid and alkali treatments on biological activity as assessed by inflammation in the mouse peritoneal cavity, the leaching of Si, and the phase contrast optical microscopy (PCOM) fiber number. We used mild and medium treatments with oxalic acid and Tris buffer and harsh treatment with concentrated HCl and NaOH. Mild oxalic acid and Tris treatment for 2 weeks had no effect on any of the end-points, but prolonging the mild oxalic acid treatment time to 2 months reduced the biological activity and the fiber number. Medium oxalic acid treatment reduced the biological activity and the fiber number and caused a loss of Si. Medium Tris alkali treatment reduced the PCOM-countable fibers and the biological activity but did not cause a substantial loss of Si. Harsh treatment with strong HCl did not affect the fiber number or cause leaching but the biological activity was reduced; strong NaOH reduced the fiber number and biological activity, and caused marked leaching of Si. The medium oxalic acid conditions (pH 1.4) were more acid than those found in lung cells but produced the same effects (reduction in fiber number and biological activity) as the more physiological mild treatment (pH 4.0), when prolonged. This study suggests that medium oxalic acid treatment can be used as a short-term assay to compare loss of Si, reduction in fiber number, and change in biological activity of vitreous fibers. Such a combination of *in vitro* and *in vivo* assays is likely to provide the best approach to assessing the complex factors involved in changes in the toxicity of vitreous fibers caused by residence in the lung. — Environ Health Perspect 102(Suppl 5):159–162 (1994)

Key words: vitreous fibers, dissolution, peritoneal cavity, inflammation

Introduction

The durability of fibers within the lung, following their deposition, is likely to be important for the subsequent development of pathological responses. In support of this contention, studies have shown leached fibers to have altered biological activity in terms of clearance (*1*) and carcinogenicity (*2*). However, most of these studies have been focused on asbestos fibers. There is a clear need for assays to assess and predict the persistence and biological activity of vitreous fibers in the various pulmonary milieus. Such assays should involve dissolution treatment followed by the measurement of a range of parameters including fiber number, fiber size, and aspects of fiber surface chemistry, any of which could be affected by the dissolution treatment. A further level of testing is needed to determine the biological activity of such leached fibers. Any dissolution-induced changes in the physicochemical parameters can then be matched against the biological activity to identify variable(s) responsible for any altered activity. The bioassay system ideally would be sensitive to the toxicity of fibers and would be a short-term test, such that it would quickly detect changes in fiber number, size, and surface reactivity. We believe that the short-term inflammatory response in the mouse peritoneal cavity fulfills these criteria for a biological test system. In this article we describe the characteristics of the assay system and its response to leached vitreous fibers.

The experiments described here are development work toward refinement of a protocol to be applied to a large range of different man-made fibers in the Colt Fiber Research Programme, for comparison with their biological activity in a range of assays.

This paper was presented at the Workshop on Biopersistence of Respirable Synthetic Fibers and Minerals held 7–9 September 1992 in Lyon, France.

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Materials and Methods

Fibers

The fibers used were Johns Manville Code 100/475 Special Purpose Glass Microfiber. The sample was generated in a 1 m^3 perspex chamber by a glass-lined, glass-tipped, propeller-driven Timbrell Dust Generator. The fibers passed through a cyclone to increase the respirable fraction and the airborne fibers were collected on an open-faced filter in the chamber.

Fiber Dissolution Treatment

To induce some degree of dissolution, mild, medium, and harsh treatments were used.

Mild. Fibers at a concentration of 0.4 mg/ml were rotated for 14 days in either oxalic acid, pH 4.0 (10^{-4} M), or Tris (tris hydroxymethyl methylamine), pH 9.0 (1.5×10^{-4}M). At the end of the treatment period the fibers were spun down, washed to neutrality, and made up to 1 mg/ml (assuming the original mass) for injection.

Medium. Fibers were treated with oxalic acid, pH 1.4, or Tris, pH 10.6 (10^{-1} M in both cases) for 21 days. At the end of the treatment, the fibers were handled as described for mild treatment.

Harsh. Fibers were treated with 1 M HCl and 1 M NaOH for 7 days and then washed and made up for injection assuming a fiber concentration equivalent to that present prior to dissolution.

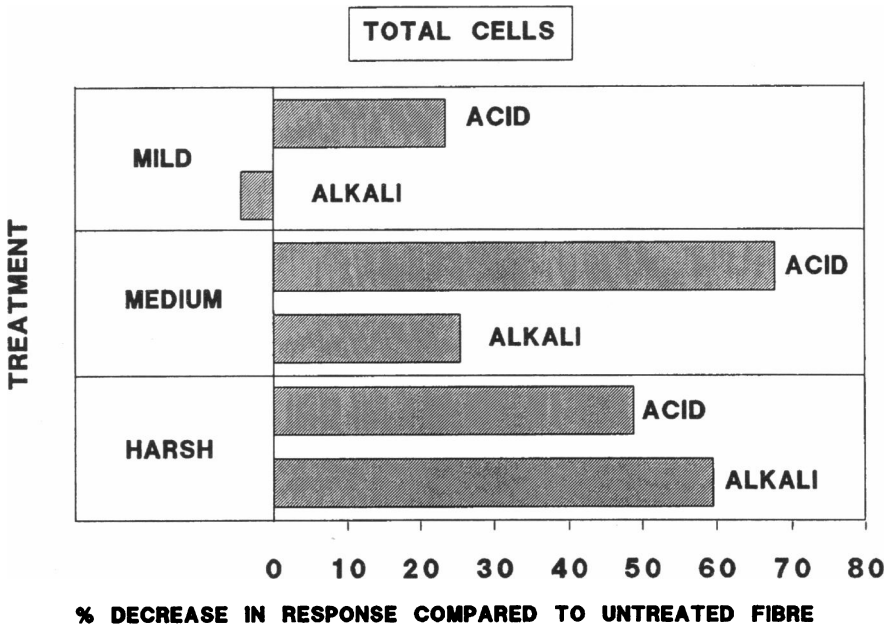

Figure 1. The effect of various treatments on the ability of Code 100/475 fibers to cause inflammation in the peritoneal cavity as measured by the total cells recruited. The scale represents the percentage decrease in response compared to untreated Code 100/475. Although mild acid caused a decrease, this did not attain statistical significance. The other decreases were significant ($p < 0.01$). Percent decrease obtained as follows:

$$\frac{(\text{Mean cells recruited in response to native fiber} - \text{Mean cells recruited in response to treated fibers}) \times 100}{\text{Mean cells recruited in response to native fiber}}$$

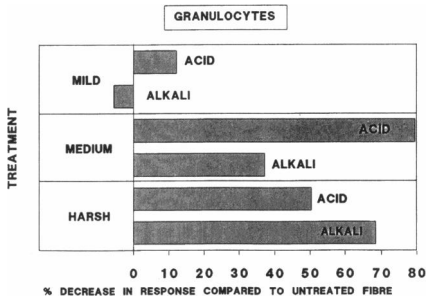

Figure 2. The effect of various treatments on the ability of Code 100/475 fibers to cause inflammation in the peritoneal cavity as measured by total granulocytes recruited. The scale represents the percentage decrease in response compared to untreated Code 100/475. Although mild acid caused a decrease, it did not attain statistical significance. Percent decrease calculated as for total cells but using the total granulocyte data.

Assessment of Fiber Number

Fiber number was determined by Phase Contrast Optical Microscopy (PCOM) according to World Health Organization (WHO) rules for regulated fibers (all fibers >5 μm in length, <3 μm in diameter and with an aspect ratio of >3:1).

Inflammation in the Mouse Peritoneal Cavity

This was assessed as described previously (3). Briefly, groups of four C57/B16 mice aged >6 weeks were instilled with 0.5 ml of saline containing 0.5 mg of fiber that had been treated in the ways described. At 4 days the peritoneal cavity was washed out with 4 × 2 ml volumes of saline and the total and differential cell count was determined. All data were expressed as millions of either total cells or granulocytes.

Measurement of Silica Dissolution from Fibers

The supernatants from treated fibers were analyzed for the presence of silica, using flame atomic absorption with a nitrous oxide acetylene flame (Thermo-electron Video 22; Warrington). Calibration solutions were prepared, using a Si standard, in the same solutions as the dissolution regimen under test (i.e., Tris buffer, oxalic acid, HCl, etc.).

Statistical Analysis

Effects of treatment were analyzed with the Minitab Statistical Package utilizing either two factor or three factor analysis of variance using the General Linear Model. The response variables were total cells from the lavage or total granulocytes, expressed in millions. In some cases the data were logarithmically ($\log_e$ or $\log_{10}$) transformed to obtain a normal distribution prior to analysis. The classifying variables in the analyses are as described below.

Results

Effect of Treatments on Inflammation in the Mouse Peritoneal Cavity

The results are presented as the mean percent decrease in total cells (Figure 1) or granulocytes (Figure 2) recruited to the mouse peritoneal cavity following instillation of treated fibers, compared to instillation of untreated fibers in the same experimental sequence. The total number of cells recruited in response to untreated fibers ranged from 6 to 12×10^6, while total neutrophils ranged from 1 to 6×10^6.

Mild. With untreated, mild oxalic acid and mild Tris alkali treatment as the classifying variables, analysis of variance showed no significant effect on the total number of cells recruited to the mouse peritoneal cavity ($F = 0.825$; $p > 0.25$) nor in the total granulocytes ($F = 1.094$; $p > 0.25$). This is shown in the top panels of Figures 1 and 2 as a slight decrease in activity with the acid treatment and a slight increase with alkali treatment.

When mild oxalic acid treatment (normally 2 weeks) was prolonged to 2 months, there was a decrease in the total fiber count (Table 1) and a decrease in the inflammatory activity.

Medium. In analysis of variance using untreated fibers or fibers following medium acid, or medium alkali treatments as classifying variables, there was a significant variance ratio of $F = 11.43$ ($p < 0.01$) for total cells and $F = 18.28$ ($p < 0.01$) for granulocytes. Individual comparisons of each leaching treatment with the controls revealed significant ($p < 0.01$) decreases in total cells recruited with acid treatment, while alkaline treatment just failed to attain statistical significance ($p > 0.05 < 0.1$; see

Table 1. Effect of increasing the treatment time with mild oxalic acid, pH 4.0, on the fiber number and inflammogenicity.

Oxalic acid treatment	Number of fibers/μg	Total granulocytes
Mild	164,239	1.51 (0.29)
Prolonged mild (2 months)	77,046	0.81 (0.10)

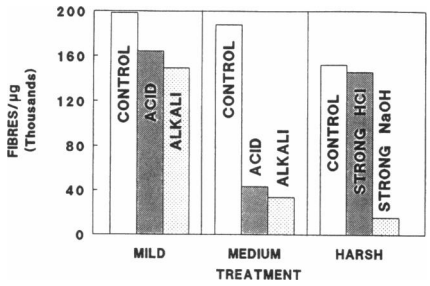

Figure 3. The total regulated fibers (>5 µm in length, <3 µm in diameter and with an aspect ratio >3:1, assessed by PCOM), following treatment with the various acid and alkali solutions; data derived as a single measurement.

Table 2. Summary of the effects of the various treatments on the inflammogenicity, loss of Si and number of PCOM-countable fibers of Code 100/475 special purpose glass fiber.

Treatment	Inflammation	Si leaching	Fibers
Mild oxalic acid	No effect[a]	+	No effect
Mild tris alkali	No effect	+	No effect
Medium oxalic acid	Reduced[b]	+++	Loss
Medium tris alkali	Reduced	+	Loss
Harsh HCl	Reduced	+	No effect
Harsh NaOH	Reduced	++++	Loss

[a] Prolonging the mild oxalic acid treatment resulted in decreases in fiber number and inflammation.
[b] The effect of medium acid was greater than the effect of medium alkalai.

Figure 1, middle panel). In the case of granulocytes, both acid ($p < 0.001$) and alkaline ($p < 0.05$) treatments significantly reduced the number of cells recruited (Figure 2, middle panel).

Harsh. Using untreated fibers, or fibers following harsh acid (HCl) or harsh alkali (NaOH) treatments as classifying variables, there was a significant effect on both total cells ($F = 6.24$; $p < 0.01$) and granulocytes ($F = 10.25$; $p < 0.01$). Individual *t*-tests confirmed that there were significant decreases compared to controls with both treatments. Significance with acid treatments for total cells and for granulocytes, $p < 0.05$; with alkali treatment for total cells, $p < 0.01$, and for granulocytes, $p < 0.001$. In general, there was a greater effect from the alkali than from the acid treatment (Figures 1, 2, bottom panels).

Effect of the Various Treatments on the Leaching of Si

All of the data from these experiments are shown in Figure 3. It is clear that only two of the treatments caused a substantial loss of Si into solution; the harsh alkali treatment caused the greatest effect, while medium acid was showing a modestly increased leaching effect at 21 days. Neither strong HCl nor medium Tris alkali was effective in causing loss of Si.

Effect of the Treatments on the Number of Fibers Countable by PCOM

Although both of the mild treatments caused a reduction of PCOM-countable fibers, this was not substantial (Figure 4). By contrast, both the medium acid and the medium alkali treatments caused a dramatic reduction in the number of fibers countable by PCOM. Fibers given harsh treatment, surprisingly, showed no effect from the acid treatment while the alkali treatment did cause marked reduction in the countable fibers (Figure 4).

Summary of Results

The results from the study are summarized in Table 2.

Discussion

The main conclusions to be drawn from the effects of the acid and alkaline treatments of special purpose glass microfiber Code 100/475 are as follows:

Neither mild Tris alkali (pH 9.0; 1.5×10^{-4} M) nor mild oxalic acid (pH 4.0; 1×10^{-4} M) treatment affected fiber size distribution, number, or biological activity; and no significant leaching of Si was detected. However, increasing the duration of the oxalic acid treatment to two months did result in a decrease both in the fiber number and in biological activity. Unfortunately there were no data available on the leaching of Si during this prolonged mild acid treatment.

Medium oxalic acid (pH 1.4; 0.1 M) and medium alkali (pH 9.6; 0.1 M) treatment for 21 days both caused a reduction in inflammatory potential, although this was greatest with the acid treatment. The effect of alkali treatment on the granulocytes was significant; it was not significant, however, on the total cell count, confirming the lesser effect of the alkali treatment on biological activity. However, both treatments substantially reduced the fibers countable by PCOM in all length categories. The Si leaching data tended to parallel the biological activity data, since oxalic acid treatment caused a loss of Si, while the leaching from Tris alkali-treated fibers was around control levels. The main discrepancy in these data relates to the alkali treatment-related reduction in PCOM-countable fibers of the same order as that seen with the acid treatment, in the absence of loss of Si and with less reduction in the biological activity than that seen with acid treatment. One explanation

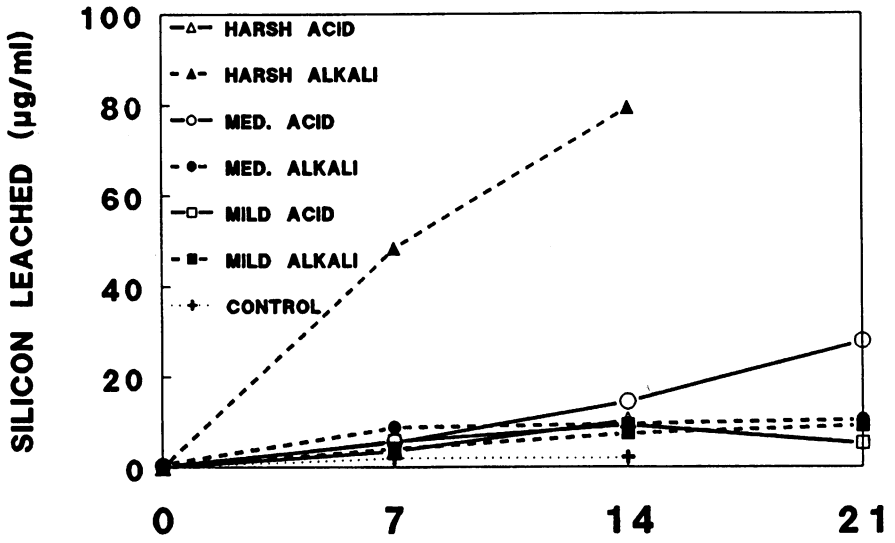

Figure 4. Leaching of silicon from fibers into the various acid and alkali solutions over time; data derived as a single measurement.

could be that alkali treatment, through loss of elements other than Si, renders the fibers less visible by PCOM, although they retain biological activity. This was, indeed, evident in the bulk solution of treated fibers, where the fibers themselves were invisible to the naked eye following alkali treatment. Additional evidence that this could be true comes from scanning electron microscope images of alkali-treated fibers, which are much reduced in their electron density.

With both harsh acid (HCl) (pH 0.1;1 M) and alkaline (NaOH) (pH 13.9;1 M) treatment for 7 days there was a reduction in biological activity. However, surprisingly, only the alkali solution caused loss of Si and loss of fibers in all size categories, whereas the strong acid (HCl) treatment caused neither leaching of Si nor a change in the PCOM-countable fiber number. This could be explained by some effects of strong HCl at the fiber surface, which prevented the process of leaching, perhaps by forming an insoluble layer that also reduced the biological activity. More work is needed to explain this phenomenon.

These results show that, for the conditions and the vitreous fiber used here, neither measuring the loss of Si into solution nor assessing the fiber size (by PCOM, at least) is a perfect descriptor of a change in biological activity, as measured by inflammation in the peritoneal cavity. For instance, strong HCl treatment caused neither leaching of Si nor a reduction in the fiber number, but the biological activity was reduced. In contrast, medium alkali treatment did not cause substantial leaching of Si from the fiber, but both the PCOM fiber counts and the biological activity were reduced.

The aim of this study was to establish the conditions suitable for a benchtop test of vitreous fiber durability, not necessarily to mimic the exact conditions in the lung. Both alkaline and acid conditions were chosen (although probably only acid conditions exist in the lung) since it has been reported that glass may be more chemically degradable at alkaline pH. In fact, the particular glass under study was degradable under both acid and alkaline conditions. The harsh treatment was included despite its extreme unphysiological nature, as a first attempt to induce rapid dissolution, used in previous studies (*2*). This yielded an interesting result, namely the failure of strong HCl to produce Si dissolution while at the same time causing a decrease in biological activity.

We demonstrated that treating the vitreous fibers in pH conditions that could obtain in the lung (mild oxalic acid for 2 weeks) produced no change in the biological activity of the fibers. However, extending that same treatment to 2 months both changed the biological activity and reduced the fiber number, an effect similar to that produced by a short-term treatment in medium oxalic acid. Medium oxalic acid treatment for 2 weeks caused differential release of Si from a range of fibers (data not shown) and this could form the basis of an *in vitro* dissolution assay. However, further research would be necessary, comparing prolonged treatment in mild oxalic acid with short-term treatment in stronger oxalic acid on a range of different fibers, before there could be confidence in the short-term assay.

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